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Award Number: W81XWH-06-1-0782

TITLE: Molecular Basis for BRCA2-mediated DNA Repair and Breast Tumor Suppression

PRINCIPAL INVESTIGATOR: Julia Etchin

CONTRACTING ORGANIZATION: Yale University
New Haven, CT06520

REPORT DATE: October 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
15-10-2007	Annual Summary	15 SEP 2006 - 14 SEP 2007

4. TITLE AND SUBTITLE
Molecular Basis for BRCA2-mediated DNA Repair and Breast Tumor Suppression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0782

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Julia Etchin

Email: julia.etchin@yale.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University
New Haven, CT 06520

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

In eukaryotes, homologous recombination and the homology-directed repair of DNA double-strand breaks are mediated by the RAD51 recombinase. In catalyzing recombination reactions, RAD51 must first form a right-handed helical filament, termed the presynaptic filament, on single-stranded DNA. Emerging evidence indicates that BRCA2 acts a recombination mediator by promoting the assembly of the RAD51 presynaptic filament. BRCA2 binds DNA and associates with RAD51. Our laboratory has established biochemical systems to examine the recombination mediator function of BRCA2. The main focus of my fellowship project is to define the role of DNA binding in this BRCA2 function. The BRCA2 DNA-binding domain (DBD) represents a highly conserved region within BRCA2-like molecules and harbors a significant portion of tumor-derived missense mutations, underscoring the importance of addressing the functional significance of this BRCA2 domain.

15. SUBJECT TERMS
DNA Repair; Tumor Suppressor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	8	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

Germ line mutations in the breast cancer susceptibility gene BRCA2 predispose carriers to early-onset breast cancer. BRCA2-deficient cells exhibit chromosomal instability and increased sensitivity to genotoxic agents. The involvement of BRCA2 in DNA double-strand break repair through the homologous recombination pathway is likely to account for these phenotypic changes, however, the mechanistic role of BRCA2 in homologous recombination remains to be defined. The main goal of this fellowship project is to define the role of DNA binding in recombination mediator function of BRCA2. Moreover, I introduce cancer-associated mutations found in the DNA-binding domain (DBD) of BRCA2 in an attempt to rationalize how mutations in BRCA2 lead to genome instability and breast cancer.

Body

To elucidate the relevance of DNA-binding by BRCA2 in the DNA homology-directed repair of chromosomal breaks and to delineate the effect of cancer mutations on this BRCA2 function, I conducted the following molecular studies proposed in the Statement of Work of my fellowship proposal.

To delineate the mode and the specificity of DNA binding by BRCA2, I purified human BRCA2 DBD and defined its DNA binding properties. I examined the affinity of BRCA2 DBD for different length DNA substrates. I conducted DNA binding with purified BRCA2 DBD. In these experiments, ^{32}P-labeled ss DNA substrates free of secondary structure (e.g. poly dT) were individually incubated with several different amounts of BRCA2 DBD, followed by analysis of the reaction mixtures in a non-denaturing polyacrylamide gel. After drying, the gel was subject to phosphorimaging analysis to detect DNA binding. The results indicate that the minimal DNA binding region of BRCA2 DBD is 24 nucelotides. Studies conducted in the Pavletich laboratory (Yang et al, 2002, 2005) have found that the mouse BRCA2-DBD and *Ustilago maydis* Brh2 protein (the BRCA2 orthologue) both bind preferentially to a duplex-ssDNA junction that harbors a 3' ss overhang. Thus, it will be of interest to investigate whether human BRCA2 DBD likewise possesses specificity for this DNA structure and additional DNA substrates that resemble HR intermediates (partial duplex, D-loop, etc.).

BRCA2 contains three canonical OB (oligonucleotide and oligosaccharide binding) folds that confer a DNA binding ability. The structure of BRCA2 DBD in complex with oligo $(dT)_9$ solved by the Pavletich group illustrates that the OB fold harbors strategically placed, conserved basic and aromatic residues that engage DNA through ionic interactions with the phosphodiester backbone and stacking interactions with bases in DNA, respectively (Yang et al, 2002). To ascertain the contribution of the BRCA2 OB folds to DNA binding affinity of BRCA2, I mutated conserved aromatics and positively charged residues in individual OB folds of the BRCA2 DBD. In OB2, W2990 intercalates between two nucleotides of the DNA ligand, thus likely representing a critical residue for DNA engagement. I introduced W2990A mutation alone and in combination with a mutation in basic residue K2833A to attenuate the affinity of OB2 for DNA.

In OB3, the invariant F3139 stacks between two nucleotides in the BRCA2 DBD-ssDNA co-crystal and K3104 is predicted to contact DNA ligand based on its orientation in the crystal structure. To inactivate the DNA binding activity of OB3, mutations in F3139 alone and in combination with K3104 and other putative DNA-binding residue Q3126 were introduced in OB3. Although no crystal structure of the OB1 fold in complex with DNA polymer exists, isolated OB1 binds DNA, suggesting that it contributes to the overall affinity of BRCA2 for DNA. To explore the contribution of OB1 to DNA binding by BRCA2, invariant residues K2777 and Y2726 were changed to alanine to create single and double mutants. To ascertain the manner in which DNA binding is affected by the mutations in OB folds, I expressed the BRCA2 DBD variants fused to a six histidine tag in bacteria. I carried out a five-step procedure - encompassing ammonium sulfate precipitation of bacterial extract, an affinity step in nickel-NTA agarose, and also chromatographic fractionation in macro-hydroxyapatite (MHAP) column to purify BRCA2 DBD variants. Although the purification scheme devised is optimal for these mutant polypeptides, the bacterial extract does not present the most suitable system for purification of BRCA2 DBD variants. Recently, a postdoctoral fellow in our laboratory successfully carried out expression and purification BRCA2 DBD in insect cell system. Thus, I am currently focusing on expression and purification of the BRCA2 DBD mutant variants to near homogeneity in insect cells. I expect to purify the mutant BRCA2 DBD variants and to assay them for DNA binding, alongside the wild type DBD, in the electrophoretic DNA mobility shift assay within the next six months.

It is possible that the point mutations within a particular OB fold in the context of DBD will not significantly diminish the DNA binding activity of BRCA2 DBD. To determine if the generated mutations are effective in ablating or substantially reducing the DNA binding affinity by BRCA2, I currently work on expression and purification of both the wild type and the mutant polypeptides of individual OB folds. The DNA binding affinity of the mutant variants will then be compared to wild type using the electrophoretic mobility shift assay. If the point mutations significantly lower the affinity of isolated OB fold for DNA, then I can conclude that particular OB fold makes little contribution to the cumulative affinity of BRCA2 DBD. If, however, the mutations introduced within isolated OB folds do not differ in the ability to bind DNA when compared to wild type variants, then additional mutations selected from candidate residues will be introduced.

A striking feature of the BRCA2 DBD is the Tower anchored on OB2. We have shown that BRCA2 DBD is capable of binding dsDNA. To investigate the contributions of the Tower domain to the DNA-binding activity of BRCA2, I deleted the individual helices of the 3-helix bundle (3HB) in the context of the BRCA2 DBD in order to purify these mutant variants to use in DNA binding experiments that employ dsDNA and DNA substrates that resemble HR intermediates.

Our laboratory is interested in the question of whether human BRCA2 has a recombination mediator activity. Due to the exceedingly large size of human BRCA2 (3,418 amino acid residues), it has not yet been possible to obtain sufficient amounts of full-length protein for mechanistic studies. Thus, our research group employed a modular approach that entails combining selected BRC repeats, BRC3 and BRC4 that are known to bind RAD51 with avidity (Chen et al, 1998; Wong et al, 1997), with the DBD of

BRCA2. The resulting polypeptide, which we refer to as BRC3/4-DBD, possesses the recombination mediator activity (San Filippo et. al, 2006).

To define the relevance of the OB folds on the recombination mediator function of BRC3/4-DBD, I introduced the polar and aromatic residues predicted to diminish DNA binding by BRCA2 within BRC3/4-DBD polypeptide in order to test the resulting polypeptides in *in vitro* homologous pairing reaction. The importance of Tower domain in recombination mediator activity of BRCA2 will be assessed in a similar experimental design. I designed constructs that carry deletions of individual helices of 3HB within the context of BRC3/4-DBD polypeptide to be tested for recombination mediator efficacy. I expect to find that attenuation of DNA binding affinity by mutating the OB folds and the Tower domain will have a significant impact on the recombination mediator function of BRC3/4-DBD.

The BRCA2 DBD represents a highly conserved region within BRCA2 orthologues and harbors a significant portion of cancer-derived missense mutations, emphasizing the importance of this region in the tumor suppressor function of BRCA2 (BIC database: Szabo et al, 2000). To examine the effect of tumor-derived DBD mutations on BRCA2-dependent recombination, I introduced the cancer-associated Tower mutations - E2856A, I2944F, K2950N, and A2951V – into BRC3/4-DBD. I will express and purify the mutant variants, and delineate their DNA binding and recombination mediator activities. The functional consequence of the cancer-associated OB2 mutations – S2988G and Q3026E – will be similarly assessed. The results will provide important molecular information to link BRCA2 mutations to the cancer phenotype.

Key Research Accomplishments

Task 1: To delineate the mode and the specificity of DNA binding by BRCA2.

- Defined the minimal DNA length required for BRCA2 binding
- Mutated conserved positively charged residues and aromatics within OB folds and the Tower domain in the context of BRCA2 DBD
- Deleted the individual helices in the 3HB of the Tower domain in the context of the BRCA2 DBD
- Devised purification scheme for the aforementioned BRCA2 DBD mutant polypeptides and identified insect cells to be optimal for expression and purification of BRCA DBD variants to near homogeneity

Task 2: Define the relevance of the OB folds and the Tower domain on the recombination mediator function of BRCA2 and examine the effect of tumor-derived DBD mutations on this BRCA2 function.

- Introduced OB fold and Tower domain mutants into BRC3/4-DBD construct for expression and purification in insect cell system
- Fused BRC3/4 region to either individual OB folds or OB folds in tandem for expression and purification in insect cell system

- Introduced the cancer-associated Tower mutations - E2856A, I2944F, K2950N, and A2951V - into BRC3/4-DBD constructs

Reportable Outcomes

Yale University provides an excellent training environment for breast cancer research, with a formal Breast Cancer Research Program (BCRP) within the Yale Cancer Center, a NCI-designated Comprehensive Cancer Center. As a component of my training, I attend monthly meetings of the BCRP and present my research findings in the spring semester.

Conclusions

The focus of my research is to delineate the importance of DNA binding by BRCA2 in its recombination mediator function. I am carrying out systematic mutational analysis of BRCA2 DBD in an attempt to rationalize how mutations that diminish DNA binding activity of BRCA2 lead to genomic instability and breast cancer. By characterizing the biochemical functions of BRCA2, we should gain a molecular understanding of why breast tumorigenesis is associated with BRCA2 mutations. The knowledge garnered from our studies could very well be exploited for the prevention, diagnosis, and treatment of breast cancer. The experimental systems that I will devise while conducting my research studies will be a valuable tool for assessing the functional consequence of BRCA2 mutations in breast cancer patients.

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